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Drosophila melanogaster* Glutamate-Cysteine Ligase Activity Is Regulated by a Modifier Subunit with a Mechanism of Action Similar to That of the Mammalian Form

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Glutamate-cysteine ligase (GCL) plays an important role in regulating glutathione homeostasis. In mammals, it comprises a catalytic (GCLC) and modifier (GCLM) subunit. The existence of a modifier subunit in invertebrates has not been described to date. We now demonstrate that GCL from *Drosophila melanogaster* has a functional modifier subunit (DmGCLM). A putative DmGCLM was obtained as an expressed sequence tag with 27% identity to human GCLM at the amino acid level. *D. melanogaster* GCLC (DmGCLC) and the candidate DmGCLM were expressed separately in *Escherichia coli*, purified, mixed, and then subjected to gel filtration, where they eluted as an ~140-kDa complex. DmGCLC co-immunoprecipitated with DmGCLM from S2 cell extracts, suggesting that they also associate *in vivo*. Enzyme kinetic analyses showed that DmGCLC has a K_m for glutamate of 2.88 mM, but when complexed with DmGCLM, the K_m for glutamate is 0.45 mM. Inhibition of DmGCLC activity by glutathione was found to be competitive with respect to glutamate ($K_i = 0.03$ mM), whereas inhibition of the GCL complex was mixed ($K_i = 0.67$ mM), suggesting allosteric effects. In accordance with this, DmGCLC and DmGCLM have the ability to form reversible intermolecular disulfide bridges. A further mechanism for control of *D. melanogaster* GCL was found to be induction of DmGCLC by *tert*-butylhydroquinone in S2 cells. DmGCLM levels were, however, unaffected by *tert*-butylhydroquinone.

Cellular injury from reactive oxygen species and electrophilic agents is inhibited by glutathione, an abundant and essential tripeptide thiol. Despite the critical importance of glutathione for virtually all aerobically respiring cells, relatively little is known about how cells regulate glutathione homeostasis. It is apparent, however, that a key player in the control of glutathione synthesis is glutamate-cysteine ligase (GCL,¹ EC 6.3.2.2, γ -glutamylcysteine synthetase), which catalyzes the first reac-

tion in the two-step pathway for *de novo* synthesis. The ultimate enzyme in the pathway, glutathione synthetase, does not appear to play such an important part in the fine-tuning of cellular redox status. GCL activity seems to be regulated by cellular thiol antioxidant balance, and in mammals, this control mechanism is principally mediated by a non-catalytic polypeptide (GCLM) that dimerizes with the catalytic subunit of GCL (GCLC) (1, 2).

Native and recombinant GCLs from rat and human have been extensively characterized (1–4). It has been shown that GCL from these species comprises an ~73-kDa catalytic subunit and an ~31-kDa regulatory subunit. GCLC has all of the catalytic activity, but *in vitro* experiments with rat kidney GCL suggested that, unless complexed with GCLM, it has exquisite sensitivity to feedback inhibition by glutathione and a high K_m (~18 mM) for glutamate (2). Combination with GCLM caused the K_i for glutathione to be substantially increased and the K_m for glutamate to be reduced by 13-fold. The presence of reducing agents caused the K_m for glutamate to increase by ~2-fold and increased the extent of inhibition by the glutathione analog ophthalmic acid, suggesting that GCLM may regulate GCLC activity in response to the immediate redox environment. Based on the *in vitro* data, it has been proposed that rat GCLC would not be functional *in vivo* without the presence of GCLM (1).

Although GCLM seems to have an important role in regulating glutathione synthesis, to date, only GCL from vertebrates has been shown to have a functional regulatory subunit. GCL from *Escherichia coli* is distinct from the eukaryotic forms described so far and is a monomer with a subunit molecular mass of 58 kDa (5). Interestingly, the *E. coli* GCL amino acid sequence has a greater level of identity to GCLM than to GCLC (1). Catalytic subunits of GCL from *Saccharomyces cerevisiae* and *Caenorhabditis elegans* have been identified (6, 7), and although they are structurally related to human GCLC (32 and 54% identities at the amino acid level, respectively), there are no convincing candidate genes in the data base with comparable levels of sequence identity to GCLM. GCL from *Trypanosoma brucei* has been cloned and its enzyme activity rigorously characterized (8, 9). Although *T. brucei* GCL shares 45% sequence identity at the amino acid level with mammalian GCLC, the kinetic data are highly suggestive that *T. brucei* GCL would be fully functional *in vivo* without the necessity for a regulatory subunit.

Recently, GCL from *Drosophila melanogaster* was cloned by functional complementation of an *S. cerevisiae gsh1* mutant (10). This was previously called *D. melanogaster* γ -glutamylcysteine synthetase, but in the present study, the nomenclature has been changed to DmGCL in accordance with the

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¹ The abbreviations used are: GCL, glutamate-cysteine ligase; GCLM, glutamate-cysteine ligase modifier subunit; GCLC, glutamate-cysteine ligase catalytic subunit; DmGCL, *D. melanogaster* glutamate-cysteine ligase; ORF, open reading frame; BSO, L-buthionine-(SR)-sulfoximine; tBHQ, *tert*-butylhydroquinone.

recommendations of IUBMB.² DmGCL was found to have 57% amino acid sequence identity to human GCLC, and its expression in the *S. cerevisiae gsh1* mutant partially restored the glutathione deficiency in this strain. The levels of glutathione in the complemented yeast strain were, however, only 8% of those in the parental strain. GCL activity of the expressed protein was not characterized in that study, but based on the high level of amino acid sequence identity to human GCLC and the fact that complementation of the *gsh1* mutant resulted in such a modest restoration of glutathione levels, it seemed to us highly plausible that DmGCL may require a regulatory subunit for efficient activity *in vivo*.

The genetic tractability of *Drosophila* makes it a highly attractive organism to study the regulation of GCL activity *in vivo*. Identification of common regulatory mechanisms for glutathione synthesis in *Drosophila* and man would make this an important model to aid our understanding of the functions of glutathione and the factors that regulate its homeostasis. In this study, we have identified a candidate GCL regulatory subunit in *Drosophila* and demonstrate that it regulates DmGCL activity in a manner similar, but not identical, to that of human or rat GCLM.

EXPERIMENTAL PROCEDURES

Construction of DmGCL Constructs—The open reading frame (ORF) of the *gcl* gene was amplified from pDmGCS4.3.3 (10) by PCR using upstream (5'-GGGAATTCATATGGGTCTACTGAGCGAGGGC-3') and downstream (5'-GCCTTAACCTCGAGTCATTTCTCTCGCAGCAGCC-3') oligonucleotides designed to insert *EcoRI* and *NdeI* sites at the 5'-end and an *XhoI* site at the 3'-end of the ORF. The amplified fragment was cloned into the *EcoRI* and *XhoI* sites of pBluescript SK⁻ (Stratagene), whereupon a 1880-bp *AccIII/XhoI* fragment was replaced with the corresponding fragment from pDmGCS4.3.3 to minimize the introduction of PCR errors. Plasmid DNA was isolated, and the insert was sequenced to confirm that it was identical to the predicted ORF of pDmGCS4.3.3. The DmGCLC ORF was subcloned into the *NdeI* and *XhoI* sites of pET15b (Novagen) to generate plasmid pETDmGCLC.

The DmGCLM cDNA was obtained as an expressed sequence tag (GH01757) from ResGen Genomics Resources (a subsidiary of Invitrogen), and upstream (5'-GGGAATTCATATGATACCGACCATAACG-3') and downstream (5'-GGAATTCCTCGAGCTAAACGCTCGAC-CTCG-3') primers were used to amplify the ORF by PCR. The oligonucleotides were designed to insert *EcoRI* and *NdeI* sites at the 5'-end and an *XhoI* site at the 3'-end of the ORF. The amplified fragment was subcloned into the *EcoRI* and *XhoI* sites of pBluescript SK⁻ and sequenced before subcloning into the *NdeI* and *XhoI* sites of pET15b to generate plasmid pETDmGCLM.

Expression and Purification of Recombinant DmGCL Proteins—pET-DmGCLC and pETDmGCLM were expressed separately in *E. coli* strain BL21(DE3). Transformed cells were grown at 37 °C with shaking in Terrific Broth (Sigma) containing ampicillin (100 µg/ml) to an A_{600 nm} of 0.5, and protein expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (1 mM). The temperature was lowered to 30 °C, and growth was allowed to continue for ~4 h before harvesting the cells. Bacterial cell pellets were resuspended in 20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 5 mM imidazole, and 0.1% (v/v) Igepal, and recombinant proteins were purified using nickel-agarose chromatography (QIAGEN Inc.) according to the manufacturer's instructions.

Gel Filtration Chromatography—Recombinant protein samples were dialyzed overnight against 3 liters of Buffer A (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM L-glutamate, and 5 mM MgCl₂) containing 10 mM glutathione. Protein was precipitated by the addition of ammonium sulfate to 90% saturation and pelleted by centrifugation. Precipitated protein was solubilized in 0.5 ml of Buffer A containing 10 mM glutathione and applied to a Sephacryl S-200 16/60 Hi-prep gel filtration column (Amersham Biosciences, Inc.) pre-equilibrated with Buffer A containing 0.2 mM dithiothreitol. Gel filtration was carried out by fast protein liquid chromatography (Amersham Biosciences, Inc.) at a flow rate of 0.5 ml/min, and fractions were collected at 1-min intervals. The eluate was monitored by continuous absorption at 280 nm, and protein concentrations in the eluted fractions were determined by the method of

Bradford (11). The column was calibrated using protein molecular mass standards (Amersham Biosciences, Inc.).

Analysis of GCL Activity—GCL activity was determined at 25 °C by a modification of the method of Seelig *et al.* (12) using cysteine as substrate instead of L-α-aminobutyrate and adapted for use on a Cobas Fara centrifugal analyzer (Roche Molecular Biochemicals, Hertfordshire, United Kingdom). Reaction mixtures (0.21 ml) contained 10 mM L-glutamate, 10 mM L-cysteine, 2 mM phosphoenolpyruvate, 0.4 mM NADH, 1 unit of lactate dehydrogenase, and 1 unit of pyruvate kinase. The reaction was initiated by the addition of ATP to a final concentration of 5 mM. *K_m* and *V_{max}* values were determined by measuring initial reaction rates at glutamate and cysteine concentrations of between 0 and 60 mM. Hyperbolic regression analysis software (43) was used to fit the Michaelis-Menten parameters using the Hanes plot. For inhibition studies with L-buthionine-(SR)-sulfoximine (BSO), GCL samples were incubated at 25 °C with 1.5 µM BSO in 100 mM Tris-HCl (pH 8.2), 20 mM MgCl₂, and 5 mM ATP for 10 min prior to kinetic analyses. GCL inhibition studies with cystamine (2,2'-dithiobis(ethylamine)) were performed by incubating GCL samples with 1.5 µM cystamine in 100 mM Tris-HCl (pH 8.2) for 10 min at 25 °C prior to kinetic analyses. For inhibition studies with glutathione, reduced glutathione was included in the standard reaction mixture using concentrations of between 0.1 and 10 mM. Inhibition constants were estimated using different concentrations of glutathione (0.1, 0.25, and 0.5 mM) with glutamate as the variable substrate. Nonlinear regression analysis of data was performed using Hyper software (13), and *K_i* values were estimated using Enzfpack (Biosoft). Lineweaver-Burk plots were used to assess the mechanisms of inhibition.

S2 Cell Culture—*Drosophila* S2 cells were a generous gift from Dr. W. Whitfield (School of Biological Sciences, University of Dundee, Dundee, UK) and were maintained in serum-free medium (Invitrogen) routinely supplemented with 2 mM L-glutamine and 5% (v/v) fetal bovine serum at room temperature.

Cells were seeded 24 h prior to treatment at a density of 1 × 10⁶ cells/ml and were treated with *tert*-butylhydroquinone (tBHQ) for 18 h. tBHQ was prepared as a 100 mM stock in Me₂SO and was diluted to the appropriate concentration in the medium. Control cells were treated with the equivalent concentration of Me₂SO. After treatment, cells were harvested and resuspended in Buffer B (50 mM Tris-HCl (pH 7.2), 20 mM sodium phosphate buffer (pH 7.0), 150 mM NaCl, and 1% (v/v) Igepal). Insoluble material was removed by centrifugation, and the soluble fractions were retained for analysis.

Immunoblotting and Immunoprecipitation—Antisera against purified recombinant DmGCLC or DmGCLM were raised in sheep using standard procedures (Scottish Diagnostics, Carlisle, UK). Western blotting was performed by the method of Towbin *et al.* (14), and the even transfer of samples was ensured by staining with Ponceau S as described previously (15). As an additional control for even protein loading and transfer, blots were also probed with a mouse monoclonal antibody raised against *Drosophila* α-tubulin (a gift from Dr. W. Whitfield). The antisera raised against the DmGCL subunits were each used at a dilution of 1:1000.

For immunoprecipitation experiments, cleared S2 cell lysates (250 µl, 0.5 mg/ml protein) were incubated with 50 µl of a protein G-agarose bead slurry (Amersham Biosciences, Inc.) and 1.5 µl of anti-DmGCLM antiserum for 1 h at 4 °C with gentle agitation. The beads were collected by centrifugation and washed three times with Buffer B, followed by a single wash with phosphate-buffered saline. Immunoprecipitates were analyzed by SDS-PAGE and Western blotting.

Statistical Analyses—Statistical analyses were performed using Student's paired *t* test.

RESULTS

Identification of a Putative DmGCLM Subunit—The amino acid sequence of DmGCLC is 57% identical to human GCLC. Phylogenetic alignment of DmGCLC with other GCL sequences shows that DmGCL has greater identity to mammalian GCL than to any other GCL subunit so far identified (10). We therefore hypothesized that DmGCL may be functionally similar to mammalian GCL, which requires a modifier subunit for regulatable and maximal activity. To test this hypothesis, we searched the *Drosophila* genome data base (16) for a GCLM candidate gene using the human GCLM cDNA sequence as a probe. The BLAST search identified a single gene within a bacterial artificial chromosome clone (BACF23F10, GenBankTM/EBI Data Bank accession number AC009846). The

² www.chem.qmw.ac.uk/iubmb/enzyme/EC6/3/2/2.html.

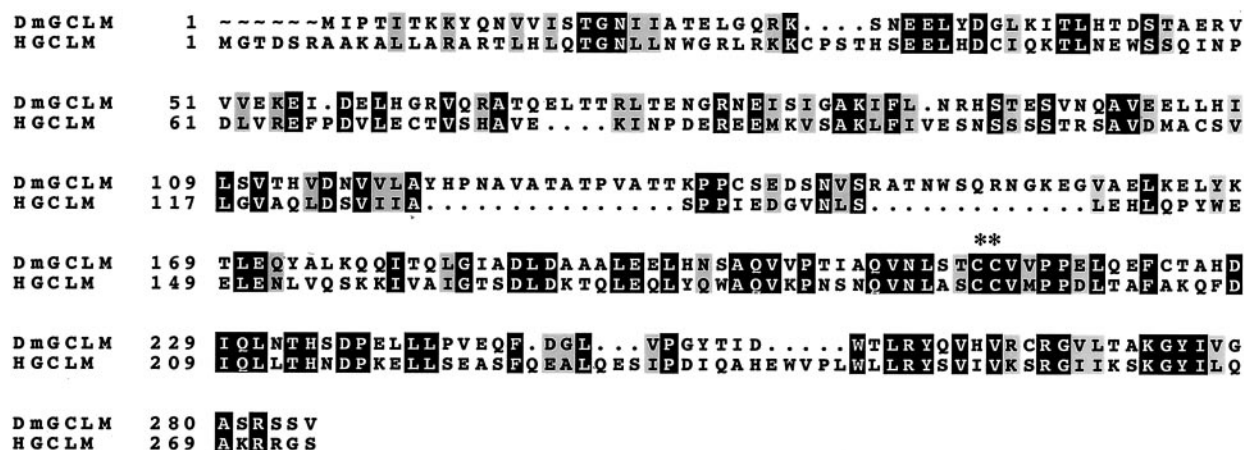


FIG. 1. Sequence alignment of DmGCLM with human GCLM. Black boxes show amino acid sequence identity. Gray boxes show amino acid sequence similarity. The cysteine residues conserved between the *Drosophila* and human (hGCLM) proteins are indicated with asterisks.

cDNA predicted from the genomic sequence encodes a 285-amino acid polypeptide with an estimated molecular mass of 31.5 kDa. The genomic sequence was used to search the *Drosophila* expressed sequence tag data base (17), and an expressed sequence tag (GH01757, accession number AI062531) with a nucleotide sequence identical to the genomic sequence was identified. The gene does not appear to have any introns over this area of the cDNA. Comparison of the predicted amino acid sequence from the putative DmGCLM cDNA with human GCLM revealed that the proteins share 27% identity and 42% similarity. The greatest level of identity is within the C-terminal region (Fig. 1).

Physical Characterization of DmGCL Subunits—The expressed sequence tag (GH01757) was used as a template to amplify the ORF of the putative DmGCLM cDNA by PCR. A single PCR product of 850 bp was obtained and subcloned into pBluescript SK⁺. The sequence was verified before the insert was subcloned into pET15b, and the resulting plasmid was used to express recombinant His-tagged DmGCLM in *E. coli* as described under "Experimental Procedures." Recombinant DmGCLM was purified from bacterial cell lysates using nickel-agarose affinity chromatography. Approximately 40 mg of recombinant protein was obtained from a 400-ml culture. Analysis of purified recombinant DmGCLM by SDS-PAGE showed that it comprises a single polypeptide with an approximate molecular mass of 31 kDa (data not shown). This is in agreement with the molecular mass calculated from the predicted amino acid sequence.

The cDNA encoding DmGCLC was isolated recently by functional complementation of an *S. cerevisiae* GCL mutant (10). In the present study, the 2160-bp ORF was cloned into pET15b and used to express DmGCLC as a recombinant His-tagged protein. This was purified from soluble *E. coli* extracts using nickel-agarose affinity chromatography, and ~20 mg of DmGCLC was obtained from an 800-ml culture. Analysis of purified recombinant DmGCLC by SDS-PAGE showed that it comprises a single polypeptide with an approximate molecular mass of 80 kDa (data not shown). This is in good agreement with the predicted molecular mass of 81 kDa from its amino acid sequence (719 amino acids).

The Putative DmGCLM Subunit Interacts with DmGCLC in Vitro—Human GCLM associates with GCLC to form a holoenzyme with an estimated size of 114 kDa (4). To determine whether the putative DmGCLM subunit identified here associates with DmGCLC to form a complex, purified DmGCLM (10 mg of protein) was mixed with DmGCLC (10 mg of protein) and subjected to gel filtration. The existence of an enzyme complex

should be evident by an increase in the molecular mass of the eluted protein, relative to DmGCLC or DmGCLM alone.

Analysis of DmGCLC (10 mg of protein) alone by gel filtration (Fig. 2) revealed that it eluted as a single peak with an estimated molecular mass of ~80 kDa. DmGCLM alone eluted as a single peak with an estimated molecular mass of ~30 kDa (Fig. 2). Resolution of the DmGCLC/DmGCLM mixture by gel filtration showed that there was one major protein peak with an estimated molecular mass of ~140 kDa and a minor protein peak with an estimated molecular mass of 30 kDa corresponding to uncomplexed DmGCLM. From these findings, we concluded that a new protein complex of a higher molecular mass is generated when DmGCLC and DmGCLM are mixed, inferring that they are interacting with each other *in vitro*. Enzyme activity was determined with the fractions collected from gel filtration of DmGCLC, DmGCLM, or the mixture of DmGCLC and DmGCLM. Fig. 2B shows that the peaks corresponding to DmGCLC and the putative DmGCLC·DmGCLM complex both have GCL activity.

To determine the composition of the 140-kDa protein, samples were analyzed by SDS-PAGE under reducing conditions and compared with purified DmGCLC and DmGCLM. Fig. 3 shows that polypeptides with molecular masses of 80 kDa (corresponding to DmGCLC) and 30 kDa (corresponding to DmGCLM) are present in the 140-kDa protein peak. Reversible disulfide bridges can form between GCLC and GCLM in the mammalian holoenzyme (12). To determine whether disulfide bonds can form between DmGCLM and DmGCLC, we dialyzed the peak fractions from the 140-kDa complex to remove dithiothreitol and subjected the protein to SDS-PAGE analysis under nonreducing conditions (Fig. 3). This showed that a higher molecular mass complex formed under nonreducing conditions with an approximate molecular mass of 140 kDa. The 140-kDa band occurred only when DmGCLC and DmGCLM were together and was absent when identically treated DmGCLM or DmGCLC samples were analyzed separately in the same way (Fig. 3). The 140-kDa band was notably absent in the corresponding samples analyzed under reducing conditions. These data are therefore highly suggestive that reversible disulfide linkages are involved in the association of DmGCLC with the putative DmGCLM subunit.

It is interesting to note that, like the complex eluted from the gel filtration column, the estimated molecular mass of the DmGCLC·DmGCLM disulfide complex is higher than the expected 110-kDa size of a homodimer. The estimated molecular mass of the DmGCL complex is more in keeping with a heterotrimeric structure. Molecular mass estimations by gel filtration

FIG. 2. Gel filtration of DmGCL subunits and the DmGCL holoenzyme. Recombinant DmGCL holoenzyme was generated by mixing 10 mg of purified DmGCLM with 10 mg of DmGCLC and concentrated by ammonium sulfate precipitation as described under "Experimental Procedures." The DmGCL holoenzyme (\square) and the uncomplexed DmGCLM (\circ) and DmGCLC (\blacktriangle) subunits were separately subjected to gel filtration chromatography. Eluted fractions were analyzed for protein concentration (A) and GCL activity (B).

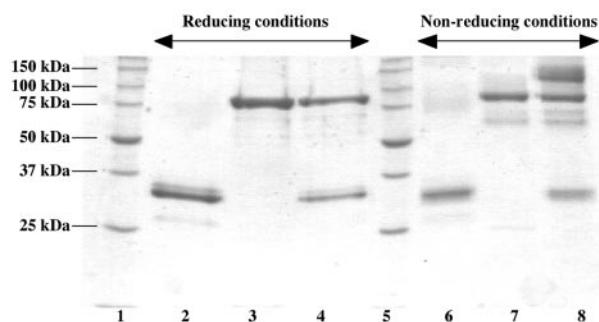
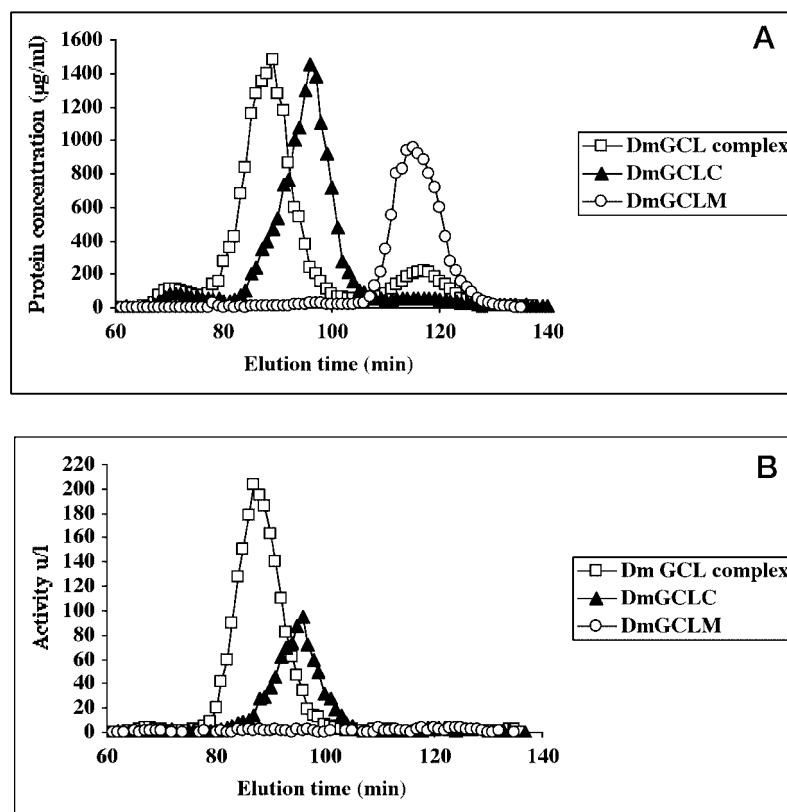


FIG. 3. Analysis of the subunit composition of the DmGCL holoenzyme. The peak fractions from gel filtration chromatography of DmGCLC, DmGCLM, or the DmGCL holoenzyme (2 µg of each) were analyzed by SDS-PAGE in the presence (lanes 2–4) or absence (lanes 6–8) of 2-mercaptoethanol. Lanes 1 and 5, molecular mass markers; lanes 2 and 6, DmGCLM; lanes 3 and 7, DmGCLC; lanes 4 and 8, DmGCL holoenzyme.

and SDS-PAGE can, however, be subject to confounding factors, and further work will be required to determine the subunit stoichiometry of DmGCL.

The DmGCLM Subunit Interacts with DmGCLC *in Vivo*—To confirm that DmGCL exists as a complex containing DmGCLC and DmGCLM *in vivo* and that our data did not result from an artifact of *in vitro* mixing, we determined whether the two subunits would co-immunoprecipitate from *Drosophila* S2 cell extracts. Soluble extracts from S2 cells were incubated with anti-DmGCLM antiserum and protein G-agarose beads, and the precipitate was analyzed by Western blotting using antiserum raised against DmGCLC. As shown in Fig. 4, a unique band was identified with an approximate molecular mass of 80 kDa when the immunoprecipitate was probed with antiserum raised against DmGCLC. This band was absent when the immunoprecipitation experiment was performed with the preimmune serum. The ability of endogenous DmGCLM to coprecipitate

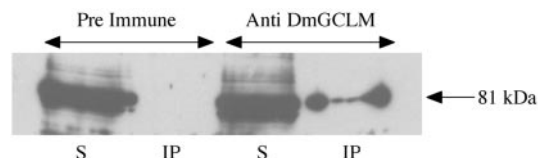


FIG. 4. Immunoprecipitation of endogenous DmGCLC from S2 cell extracts. Immunoprecipitation of DmGCLM from S2 cell extracts with antiserum raised against recombinant DmGCLM was performed as described under "Experimental Procedures." Immunoprecipitated proteins were analyzed by Western blotting using antiserum raised against recombinant DmGCLC. S, supernatant fraction remaining after immunoprecipitation; IP, immunoprecipitate.

tate with endogenous DmGCLC from S2 cell extracts implies that DmGCLM and DmGCLC form a complex *in vivo* as well as *in vitro*.

DmGCLM Modulates DmGCLC Activity—Evidence suggests that mammalian GCLM can enhance the catalytic efficiency of GCLC by increasing its affinity for its substrate L-glutamate (1, 2). To determine whether DmGCLM has a similar effect on DmGCLC activity, we compared the activities of DmGCLC and the 140-kDa DmGCL holoenzyme after purification by gel filtration.

DmGCLC and the DmGCL holoenzyme were found to have specific activities of 244 and 569 nmol/min/mg, respectively (calculated with respect to the amount of DmGCLC in the assay mixture) when measured using standard assay conditions with 10 mM L-glutamate. Activity was not detected in the absence of L-glutamate or L-cysteine. When L- α -aminobutyrate was used as substrate in place of cysteine, activity was comparatively low, even when high concentrations were used. K_m and V_{max} values were determined for L- α -aminobutyrate, and the K_m was found to be between 4- and 12-fold higher (Table I) than that reported for human GCL (3, 4). The catalytic efficiency of DmGCL (k_{cat}/K_m) with L- α -aminobutyrate as substrate is $0.53 \text{ min}^{-1} \text{ mM}^{-1}$, implying that L- α -aminobutyrate is

TABLE I
 Kinetic constants for *Drosophila* GCL

 Kinetic constants for DmGCL are compared with those for DmGCLC and were analyzed using Student's paired *t* test. AB, L- α -aminobutyrate.

Enzyme	K_m			k_{cat}/K_m			K_i for glutathione
	L-Glu	L-Cys	AB	L-Glu	L-Cys	AB	
		mM			min ⁻¹ mM ⁻¹		mM
DmGCLC	2.88 \pm 0.08	5.53 \pm 0.72	12.35 \pm 4.13	5.62 \pm 0.29	3.07 \pm 0.22	0.26 \pm 0.07	0.03 \pm 0.01
DmGCL	0.45 \pm 0.02 ^a	6.55 \pm 0.41	13.03 \pm 2.94	100.08 \pm 2.38 ^a	9.94 \pm 0.24 ^a	0.53 \pm 0.1	0.67 \pm 0.33 ^a

^a Significant differences (*p* < 0.05).

a poor substrate for DmGCL.

K_m and V_{max} values for L-glutamate and L-cysteine were determined. The apparent K_m of the DmGCL holoenzyme for L-glutamate is 0.45 mM, considerably lower than that determined for DmGCLC, which is 2.88 mM (Table I). K_m values for cysteine were found to be 6.55 and 5.53 mM for the DmGCL holoenzyme and DmGCLC, respectively. These values are substantially higher than the corresponding values for human GCL, for which values of between 0.1 and 0.8 mM have been reported (3, 4). We also examined the possibility that other amino acids could substitute for cysteine in the enzyme assay and tested a range of amino acid substrates, including methionine, alanine, serine, glycine, leucine, and lysine, in place of cysteine. We were unable to detect any GCL activity over background levels (data not shown), suggesting that these amino acids cannot be utilized instead of cysteine by DmGCL.

The k_{cat}/K_m values of the holoenzyme for L-cysteine and L-glutamate are significantly higher than those of DmGCLC alone (Table I). These findings indicate that the holoenzyme is catalytically more efficient than DmGCLC and show that DmGCL is catalytically similar to mammalian GCL in that GCLM influences GCLC enzyme activity.

DmGCLM Alters the Susceptibility of DmGCLC to Inhibition—Mammalian GCL activity can be inhibited by glutathione (2). The nature and extent of inhibition are modulated by the presence of the regulatory subunit. To determine whether a similar mechanism of regulation of GCL activity exists for DmGCL, we first examined the effect of increasing concentrations of glutathione on GCL activity under standard assay conditions (Fig. 5). DmGCLC activity was reduced by 60% in the presence of 1 mM glutathione and by 93% in the presence of 2 mM glutathione. By contrast, the holoenzyme was less susceptible to inhibition by glutathione, with 1 and 2 mM glutathione lowering activity by 32 and 58%, respectively. Higher concentrations of glutathione (10 mM) almost completely abolished the activities of both DmGCLC and the DmGCL holoenzyme. The kinetics of glutathione inhibition were studied by measuring DmGCL reaction velocities with increasing concentrations of glutamate in the presence of fixed glutathione concentrations (0.1, 0.25, and 0.5 mM). DmGCLC was inhibited competitively by glutathione when glutamate was the variable substrate. By contrast, the holoenzyme was subjected to mixed inhibition by glutathione. The apparent inhibition constant (K_i) values for glutathione were 0.03 mM for DmGCLC and 0.67 mM for the DmGCL holoenzyme (Table I). These findings show that DmGCLM significantly reduces the sensitivity of the catalytic subunit to inhibition by glutathione, possibly by generating a conformational change preventing access of glutathione to the active site in a manner similar to the model proposed by Meister and co-workers (2).

BSO is an inhibitor of GCL and is phosphorylated by the enzyme to form an intermediate that is tightly and irreversibly bound at the active site (18, 19). It was of interest to examine the effect of BSO on the DmGCLC subunit and the DmGCLC-DmGCLM complex. Incubation of DmGCLC with 1.5 μ M BSO for 10 min prior to kinetic analysis caused a 47%

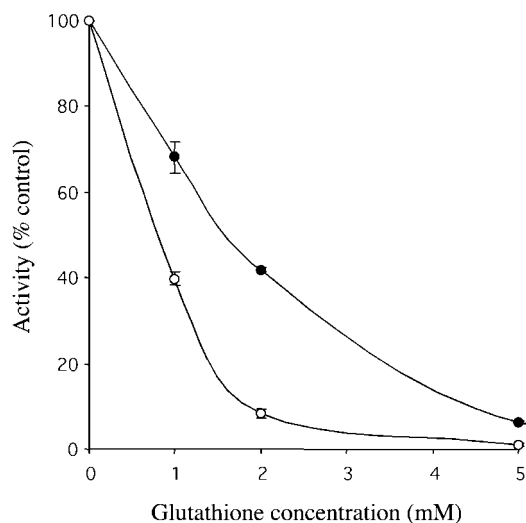


FIG. 5. **Inhibition of recombinant DmGCL activity by glutathione.** The specific activities of the DmGCL holoenzyme (●) and DmGCLC (○) were measured using standard assay conditions in the presence of increasing concentrations of glutathione. Activity is expressed as a percentage of the control activity in the absence of glutathione (mean \pm S.E.).

reduction in GCL activity, whereas DmGCL holoenzyme activity was reduced by 90% (Table II), showing that the DmGCLC-DmGCLM complex is more readily inhibited by BSO compared with DmGCLC.

Mammalian GCL has also been shown to be susceptible to inhibition by cystamine, which is thought to inhibit GCL activity by binding a cysteine residue in or around the active site (20–22). Inhibition can be reversed by treatment with dithiothreitol. Incubation of DmGCLC for 10 min prior to kinetic analysis with 1.5 μ M cystamine reduced DmGCLC activity by 34%, suggesting that the DmGCLC active site may be structurally similar to mammalian GCL with a cysteine residue near the active site (Table II). Curiously, the DmGCL holoenzyme was unaffected by the presence of 1.5 μ M cystamine. It is possible that free thiol groups on the DmGCLM subunit may preferentially form disulfides with cystamine, sparing the cysteine that is close to the active site. This raises the possibility that the GCL regulatory subunit may have an additional function to protect the catalytic subunit from thiol-reactive agents.

DmGCL Subunit Levels Are Modulated by Oxidative Stress in S2 Cells—GCLC and GCLM protein levels have been shown to increase in response to agents capable of generating sublethal oxidative stress in human cell lines (23–25). We wished to determine whether induction of the DmGCL subunits constitutes part of the adaptive response to oxidative stress in *Drosophila* and treated S2 cells with the redox cycling agent tBHQ to examine this possibility.

Western blot analyses of S2 cells treated with increasing concentrations of tBHQ showed that tBHQ caused a concentration-dependent increase in intracellular DmGCLC protein lev-

TABLE II
Effect of BSO or cystamine on DmGCL activity

The specific activities in the presence of BSO or cystamine are compared with the appropriate control activities and were analyzed using Student's paired *t* test.

Enzyme	Activity		
	No inhibitor	1.5 μ M BSO	1.5 μ M cystamine
DmGCLC	100	53.32 \pm 0.55 ^a	66.27 \pm 0.32 ^a
DmGCL	100	9.63 \pm 0.9 ^a	98.90 \pm 0.75

^a Significant differences (*p* < 0.05).

els (Fig. 6A). Treatment with 50 or 100 μ M tBHQ caused increases in DmGCLC of ~3- and 4-fold, respectively. By contrast, DmGCLM protein levels did not appear to be substantially increased by treatment with tBHQ (Fig. 6B).

DISCUSSION

GCL activity in mammals is subject to intricate regulation involving both post-translational and transcriptional control mechanisms (19). It was unknown whether these regulatory features are conserved in invertebrates, and in this study, we have demonstrated a parallelism in the regulation of GCL activity in mammals and *Drosophila*. We have shown that DmGCL is composed of a catalytic subunit and at least one regulatory subunit that enhances the affinity of DmGCLC for glutamate and reduces its susceptibility to inhibition by glutathione. Furthermore, as has been shown for human GCLC, DmGCLC protein levels can be up-regulated in cell lines by agents that generate oxidative stress.

Non-mammalian GCL has been described for a wide variety of eukaryotic species, including yeast, Nematoda, Protozoa, and Insecta, and has been isolated as a single chain polypeptide with similarity to human GCL (6–8, 10, 26–28). By contrast, *Arabidopsis thaliana* GCL has no significant identity (15–19%) to other GCL forms (29, 30). Until now, functional non-mammalian GCLM homologs have not been described in the literature. It has been suggested that certain lower eukaryotic species do not require a GCLM subunit, as enzyme kinetic studies showed that they are likely to be active *in vivo* without the requirement for a regulatory subunit (8). The lack of documentation about GCLM homologs in lower eukaryotes does not, however, confirm that they are absent. It is possible that, in certain lower eukaryotes, GCL enzymes contain functional GCLM subunits, but their presence has been overlooked due to the method of GCL isolation. Very few GCL enzymes have been isolated from invertebrates by purification of native proteins. Instead, direct cloning or functional complementation of mutants has been used to identify cDNAs encoding proteins with GCL activity. As GCLM is not required for activity *in vitro*, a requirement for its presence *in vivo* may not have been noted. The possibility that GCL may comprise catalytic and modifier polypeptides in invertebrates other than *Drosophila* is strengthened by work by Hussein and Walter (31), who purified GCL from the nematode *Ascaris suum*. Purification of *A. suum* GCL by gel filtration showed the presence of two protein peaks with GCL activity with molecular masses of 100 and 70 kDa. Although the 100-kDa protein was not characterized, the possibility that it contains catalytic and regulatory subunits would be interesting to investigate further.

Although we have raised the possibility that GCL may comprise catalytic and regulatory subunits in other invertebrates, we do not suggest that this would necessarily occur in all cases. Indeed, GCL from *T. brucei* has a K_m for glutamate of 0.24 mM and a K_i for glutathione of 1.1 mM, which are similar to those obtained for the DmGCLC·DmGCLM complex. The kinetics of

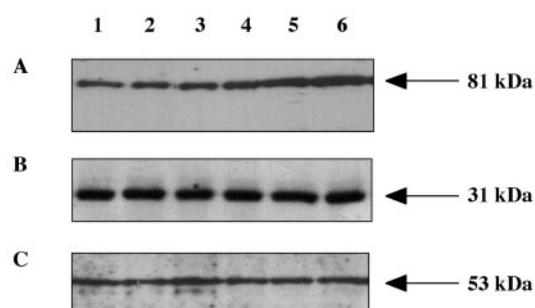


FIG. 6. Effect of tBHQ on DmGCL subunit levels in S2 cells. S2 cells were cultured in the presence of tBHQ for 18 h, and cell lysates (30 μ g of protein) were analyzed by Western blotting using antibodies raised against DmGCLC (A), DmGCLM (B), or *Drosophila* α -tubulin (C). The lanes were loaded with extracts from cells that had been treated as follows. Lane 1, no treatment; lane 2, Me₂SO; lane 3, 25 μ M tBHQ; lane 4, 50 μ M tBHQ; lane 5, 75 μ M tBHQ; lane 6, 100 μ M tBHQ. The relative intensities of cross-reactive bands were examined by scanning densitometry.

T. brucei GCL activity suggest that it may not require further activation by a GCLM subunit (8). It remains to be determined whether the regulation of GCL activity by a modifier subunit is a common feature in different invertebrates and when, in evolutionary terms, it became an important regulator of glutathione synthesis.

DmGCLM reduces the K_m of DmGCLC for glutamate and raises the K_i for glutathione in a manner similar to that of its mammalian counterparts. GCLM-mediated changes in the kinetic efficiency of mammalian GCLC are thought to be due to a conformational change in GCLC favoring a glutamate-binding site with high affinity and specificity for L-glutamate; the high affinity glutamate-binding site is also less accessible to glutathione (2). This conformational change is thought to result, in part, from the formation of intersubunit disulfide bridges between GCLM and GCLC. These covalent linkages are susceptible to changes in the reducing environment and are proposed to modify GCL activity in response to changes in intracellular glutathione concentrations (2). Our kinetic studies on inhibition of DmGCL by glutathione show that similar regulatory mechanisms for GCL activity may exist in *Drosophila*. DmGCLC inhibition by glutathione is competitive with respect to glutamate, whereas inhibition of the holoenzyme by glutathione can be classed as mixed. These findings are in keeping with the hypothesis that reduction of disulfide bridges between DmGCLC and DmGCLM by glutathione facilitates access of glutathione to the active site, where it could compete with glutamate. When DmGCLM is absent, the active site of DmGCLC appears to be accessible for competitive inhibition by glutathione. Our hypothesis that inhibition of the DmGCL holoenzyme by glutathione is likely to involve reduction of disulfide linkages between the subunits is supported by the demonstration that the DmGCLC·DmGCLM complex can form reversible disulfide linkages when subjected to SDS-PAGE under nonreducing conditions (Fig. 3). Comparison of the amino acid sequences of DmGCLM and human GCLM shows that there are two conserved cysteine residues (Fig. 1, asterisks). It is possible that one or both of these residues mediate the covalent interactions with DmGCLC, and we are currently investigating this further.

In addition to examining the effect of the modifier subunit on the catalytic efficiency of GCLC and its inhibition by glutathione, we also investigated whether GCLM influenced susceptibility to inhibition by BSO or cystamine. DmGCLM appears to protect DmGCLC against inhibition by cystamine, as DmGCLC was inhibited by cystamine, whereas the DmGCL holoenzyme was unaffected under the assay conditions used. The mecha-

nism for this protection is unclear, but it is possibly due to DmGCLM buffering the local cystamine concentrations by providing additional thiol-binding sites. Cystamine forms mixed disulfides with GCLC and binds a cysteine residue close to the active site, blocking substrate access (9, 21). It is possible that surface sulfhydryl residues on DmGCLM become preferentially bound by cystamine, thus protecting a critical cysteine at or near the active site of DmGCLC. By contrast, the DmGCL holoenzyme was found to be more sensitive to inhibition by BSO compared with DmGCLC. It has been shown that phosphorylation of BSO by GCL generates an intermediate that binds tightly and irreversibly to the glutamate- and cysteine-binding sites of GCL (32). Although we have not investigated the mechanism for the differential inhibition by BSO, it is possible that the conformational change imposed upon the active site of DmGCLC by DmGCLM generates a binding pocket with greater affinity for BSO, thus making it a more potent inhibitor for the holoenzyme than the catalytic subunit alone. Alternatively, as BSO is a time-dependent inhibitor, the differential inhibition could reflect an increase in the rate of BSO phosphorylation in the presence of the regulatory subunit.

The apparent size of the DmGCL holoenzyme complex appears to be larger than that described for rat or human GCL. We estimate the DmGCL complex formed *in vitro* to be ~140 kDa. This suggests that the reconstituted DmGCL holoenzyme may, in fact, comprise one catalytic subunit and two regulatory subunits. Studies with GCL from rat kidney led Sekura and Meister (33) and Seelig *et al.* (12) to suggest that it exists as a heterodimer. Other studies with recombinant human GCL are also consistent with a heterodimeric structure (3, 4). It has, however, been documented that the amount of GCLM associated with GCLC may vary between different enzyme preparations (34). Interestingly, GCL purified from rat liver by Davis *et al.* (35) was estimated by gel filtration to be 138 kDa, which would be consistent with a heterotrimeric structure. It is possible that the molar ratios of the catalytic and modifier subunits may be subject to a degree of variation *in vivo*.

Elevated intracellular glutathione levels and glutathione-metabolizing enzymes have been observed in mammalian cells and yeast as part of an adaptive response to oxidative stress (23, 24). Glutathione-depleting agents, heavy metals, redox cycling chemicals, inflammatory cytokines, chemotherapeutic drugs, and ionizing radiation have been shown to modify intracellular glutathione and GCL protein levels. This appears to be due to induction of GCL transcription as well as mRNA stabilization. The redox cycling agent tBHQ has been used in many mammalian cell lines to generate oxidative stress and has been shown to elevate GCL subunit mRNA levels by increasing gene transcription, leading to increased GCL protein levels, GCL activity, and elevated glutathione levels (23, 24). In this study, we found that DmGCLC protein levels were up-regulated in *Drosophila* S2 cells in response to tBHQ. Although we did not determine whether transcriptional activation of DmGCLC mRNA occurred in this study, it is likely that the elevation of DmGCLC protein levels reflects increased DmGCLC gene transcription. We have identified several putative enhancer elements in the genomic DNA sequence (16) upstream from the DmGCLC ORF. These include AP-1- and nuclear factor- κ B-binding sites and could potentially be involved in increasing DmGCLC transcription in response to tBHQ. Oxidative stress is thought to induce transcription of mammalian glutathione-associated genes by triggering signaling cascades that activate various transcription factors such as Nrf2, AP-1, and nuclear factor- κ B. Homologs of mammalian c-Jun and c-Fos as well as nuclear factor- κ B exist in *Drosophila* (D-Jun, D-Fos, and the Rel family, respectively) (36, 37), but their role in adaptation to

oxidative stress within *Drosophila* has not, to our knowledge, been characterized as rigorously as that of their mammalian counterparts.

In contrast to our findings for DmGCLC, DmGCLM protein levels were only marginally enhanced in response to tBHQ treatment. In mammals and mammalian systems, GCLC and GCLM are also subject to a degree of differential regulation, although both subunits are usually increased in response to oxidative stress (23, 24).

As has been shown for the human and mouse GCL genes (38), the DmGCL genes are on separate chromosomes. The DmGCLC gene is on the X chromosome (polytene map position 7CD) (10), whereas the DmGCLM gene maps to 94C on the third chromosome. One of the advantages of working with the *Drosophila* system is the existence of mutant stocks created by P-element mobilization (39, 40). Stocks are often in existence with mutations in or near the gene of interest. We have obtained the P-element-induced recessive lethal *l(3)Lo580*, which contains a P-element insertion in the 5'-noncoding region of the DmGCLM gene. Although we have yet to establish that the P-element insertion is responsible for the lethality, there is a good possibility that DmGCLM is essential for glutathione synthesis *in vivo*, as proposed by Meister and co-workers (1) for rat GCL from their *in vitro* studies. Targeted deletion of the GCLC gene in the mouse is lethal, showing that glutathione is essential for normal embryonic development (41, 42). To date, corresponding models for the GCLM gene have not been described. If, as we suspect, loss of DmGCLM gene function is lethal, mutants will provide valuable genetic models with which to study the regulation of GCL activity *in vivo*.

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